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Tuber microverrucosum and *T. huizeanum* — two new species from China with reticulate ascospores

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Abstract — Two new species of *Tuber* are described and illustrated. *Tuber microverrucosum* is recognized by its brown verrucose ascomata, blackish gleba, and broadly ellipsoid to subglobose ascospores with a large-meshed regular reticulum. *Tuber huizeanum* is diagnosed by its brown glabrous ascomata, brown gleba, and broadly ellipsoid ascospores with a regular reticulum. Molecular phylogenetic analysis supports the erection of the two new species.

Key words — *Ascomycota*, truffle, taxonomy

Introduction

Truffles are abundant in some regions of China (Garcia-Montero et al. 2010, Wang & Liu 2011, Chen et al. 2011a,b) from which more than 20 species have been described during the 25 years since Liu described *Tuber taiyuanense* B. Liu in 1985 (Liu 1985; Wang 1988; Wang & Li 1991; Wang et al. 1998; Wang & He 2002; Tao et al. 1989; Chen et al. 2005; Chen & Liu 2007). Of these more than half are considered valid species (Cao 2010). That there are likely more new species awaiting discovery in China is confirmed by our ongoing research (Fan et al. 2011, 2012a,b). We describe and illustrate here two additional new species from Yunnan.

Materials & methods

Morphological studies

Truffles were collected from Huize County and a mushroom market in Kunming, Yunnan Province. Macroscopic characters were described from fresh specimens. Microscopic characters were described from razor-blade sections of fresh specimens

Table 1. *Tuber* specimens used in molecular studies

Species name	Voucher specimen	Country of origin	Reference	ITS
T. borchii Vittad.	GB62	Italy	Bonito et al. 2010	HM485342
	GB45	Italy	Bonito et al. 2010	HM485344
T. canaliculatum Gilkey	JT23942	USA	From GenBank (unpublished)	GQ221456
	OSC59072	USA	Bonito et al. 2010	HM485347
T. foetidum Vittad.	ZB-2489	Hungary	Halász et al. 2005	AJ557544
	ZB3454	Finland	Orczán et al. 2010	FN568055
T. gardneri Gilkey	TK1779	USA	From GenBank (unpublished)	AY558808
T. huizeanum	BJTC FAN144	China	This paper	JN870100
	BJTC FAN186	China	This paper	JQ910651
T. linsdalei Gilkey	L63	USA	Bonito et al. 2010	HM485370
T. liui A.S. Xu	HKAS 48269	China	Chen & Liu 2007	DQ898182
T. maculatum Vittad.	FHS-390	Serbia	Marjanović et al. 2010	FM205649
	K(M) 17936	—	Brock et al. 2009	EU784428
T. microverrucosum	BJTC FAN142	China	This paper	JN870099
T. puberulum Berk. & Broome	TL11885	Denmark	Tedersoo et al. 2006	AJ969626
	BI-32	Hungary	Halász et al. 2005	AJ557537
T. separans Gilkey	ZB-32	Hungary	Halász et al. 2005	HM485388
	JT32463	Mexico	From GenBank (unpublished)	GQ221448
T. scruposum R. Hesse	CMI-UNIBO 2192	Armenia	From GenBank (unpublished)	DQ011846
	CMI-UNIBO 2201	Armenia	From GenBank (unpublished)	DQ011845
T. zhongdianense X.Y. He et al.	HKAS 45388B	China	Chen & Liu 2007	DQ898186
	Wang0299	China	Chen & Liu 2007	DQ898187
T. melanosporum Vittad.	A59	France	From GenBank (unpublished)	AF106878
	—	—	Roux et al. 1999	AF132501

mounted in 3% KOH, Melzer's reagent, or 0.1% (w/v) cotton blue in lactic acid. The specimens are deposited in BJTC (Biology Department Herbarium, Capital Normal University). For scanning electron microscopy (SEM), spores were scraped from the dried gleba, placed onto doubled-sided tape, mounted directly on an SEM stub, coated with gold-palladium, and then examined and photographed with a HITACHI S-4800 SEM.

Molecular methods

Herbarium samples were crushed by shaking for 3 min at 30 Hz (Mixer Mill MM 301, Retsch, Haan, Germany) in a 1.5 ml tube together with one 3 mm diameter tungsten carbide ball. Total genomic DNA was then extracted using the PeqLabE.Z.N.A._Fungal DNA kit following the manufacturer's protocol. The ITS region was amplified with PCR using the primers ITS1/ITS4 (White et al. 1990). PCR was performed in 50 µl reactions containing DNA template 2 µl, primer (10µM) 2 µl each, 2 × Master Mix (Tiangen Biotech (Beijing) Co. Ltd.) 25 µl. PCR reactions were run as follows: an initial denaturation at 95°C for 3 min, followed by 30 cycles at 95°C for 2 min, 55°C for 25 s, 72°C for 2 min, and a final extension at 72°C for 10 min. The PCR products were sent to Invitrogen Biotechnology Co. Ltd. (Beijing, China) for purifying, sequencing, and editing. The other sequence data of ITS rDNA included in this study were downloaded from GenBank. GenBank numbers are shown in Table 1.

Phylogenetic analyses

DNA sequences were aligned with Clustal X (Thompson et al. 1997) and manually adjusted with Se-Al v.2.03a (Rambaut 2000). The aligned dataset was analyzed with maximum parsimony (MP) using PAUP*4.0b10 (Swofford 2002). MP analysis was conducted using heuristic searches with 1000 replicates of random-addition sequence, tree bisection reconnection (TBR) branch swapping algorithm. All characters were equally weighted and unordered. Gaps were treated as missing data to minimize homology assumptions. A bootstrap (BS) analysis was performed with 1000 replicates, each with 10 random taxon addition sequences. TBR branch swapping was employed. The Bayesian analysis was performed with MrBayes 3.1.2 (Huelsenbeck et al. 2001; Ronquist & Huelsenbeck 2003) with two sets of four chains (one cold and three heated) and the stoprule option in effect, halting the analyses at an average standard deviation of split frequencies of 0.01. The sample frequency was set to 100, and the first 25% trees were removed as burn-in. Bayesian posterior probabilities (PP) were obtained from the 50% majority rule consensus of the remaining trees. Two sequences derived from *Tuber melanosporum* (AF132501, AF106878) were used as outgroups.

Results

Molecular phylogenetics

343 of 634 characters were found to be parsimony-informative. Maximum parsimony analysis produced one most parsimonious tree (Fig.1) with a length (TL) of 907 steps, consistency index (CI) of 0.7310, retention index (RI) of 0.8501, and rescaled consistency index (RCI) of 0.6214 (for all sites).

ITS sequence-based phylogenies (Fig. 1) group the new *Tuber microverrucosum* in a clade with *T. foetidum* with moderate support (BS = 74, PP = 0.99). The two *T. huizeanum* sequences grouped in a clade with strong support (BS = 99, PP = 1.00) and were sister (but with very low support) to *T. zhongdianense*.

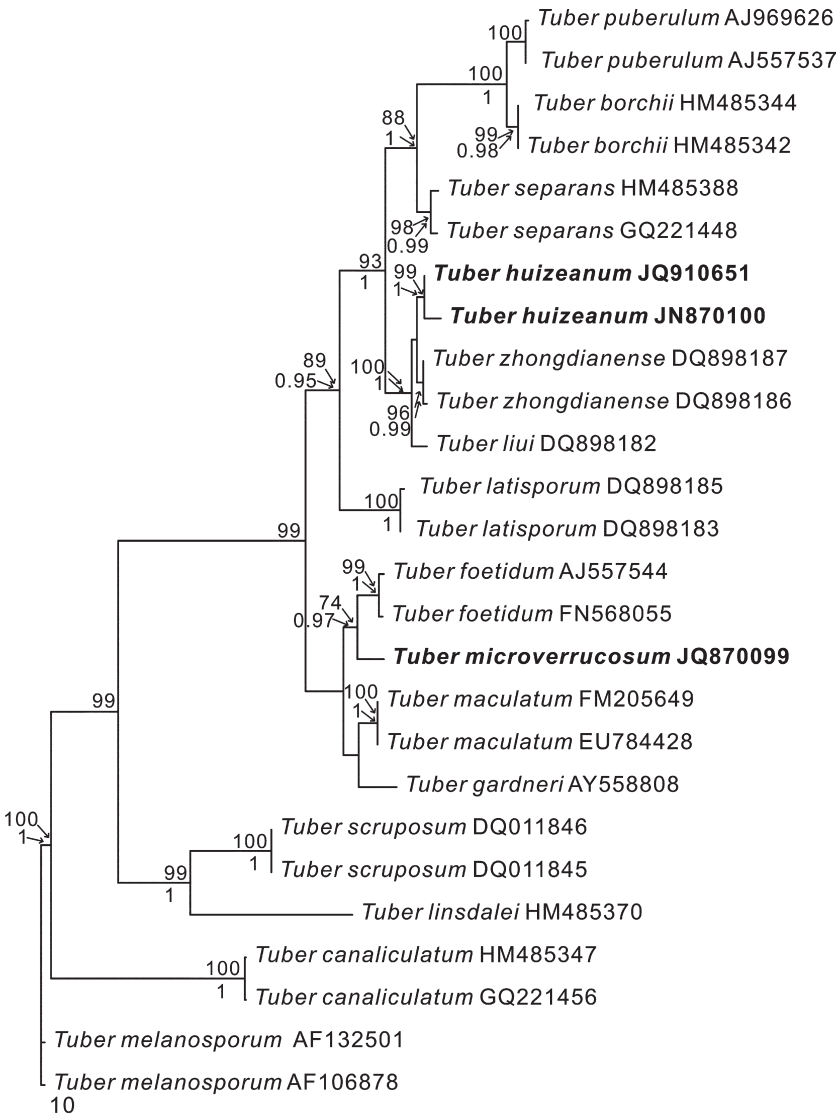

Fig. 1. Phylogeny derived from maximum parsimony analysis of the ITS rDNA sequences of some *Tuber* species with reticulate ornamentations on the ascospore surface, using *T. melanosporum* as outgroups. Bootstrap values of more than 70% (BP ≥ 70) from 1000 replications are shown above the respective branches. Bayesian posterior probabilities (PP) were estimated and clades with PP > 0.95 (95%) are marked under the branches.

Taxonomy

Tuber microverrucosum L. Fan & C.L. Hou, **sp. nov.** Fig. 2 a–d

MycoBank MB 563657

Differs from other *Tuber* species with reticulate ascospores by its brown verrucose ascomata, blackish gleba, and broadly ellipsoid ascospores.

Type: China. Yunnan Province, Kunming, from the Kunming mushroom market. 20 Jan. 2011, Jin-Zhong Cao 105 (**Holotype**, BJTC FAN142).

Etymology: *microverrucosum* (Lat.), referring to the minute verrucose surface of the ascomata.

Ascoma globose or irregular subglobose, 2.2 cm in diam., light brown to brown when fresh, the surface glabrous and distinctly minute verrucose, the places without verrucose show gray-brown or pale gray. Odor slight when young and middle pungent after maturity. Peridium 250–400 μm thick including the verrucose, two layers; outer layer pseudoparenchymatous, composed of subglobose and angular cells (7.5–)10–20(–25) μm in diam, with slightly thickened walls, yellowish-brown towards the surface; inner layer texture intricate, the hyphae hyaline and thin walled, 5–7.5 μm in diam. Gleba white at first, then brown to dark brown and blackish at maturity, marbled with larger and rare pale white veins which originate from all of the peridium. Asci ovate or subglobose, sessile, 75–100 × 50–75 μm, (1–)2–4(–6)-spored. Ascospores broadly ellipsoid to subglobose, a few globose, at first hyaline, becoming brown to dark brown at maturity, thick-walled 2.5–3 μm, surface ornamented with regular reticulum, 4–6(–7.5) μm high, 25–32.5 × 20–27.5 μm, Q = 1.10–1.25, in 2–6-spored asci, and 37.5–42.5 × 27.5–32.5 μm, Q = 1.30–1.36, in 1-spored asci, excluding the ornamentation, the meshes large and variable in size, usually 3–4 meshes across the width.

Comments — *Tuber microverrucosum* is distinguished by its brown ascomata with minutely distinct verrucose surface, blackish gleba, and broad ellipsoid to subglobose ascospores covered with regular deep reticulum.

Two European species, *Tuber foetidum* and *T. scruposum*, are similar to the new species. *Tuber foetidum* differs in having a smooth or indistinctly verrucose peridium, a pale brown gleba and yellow-brown ascospores (Pegler 1993), whereas *T. scruposum* has a distinct verrucose peridium, and narrow elliptical ascospores (Lange 1956).

Several North American species that also have verrucose ascomata and reticulate ascospores include *T. canaliculatum*, *T. linsdalei*, *T. longisporum* Gilkey, and *T. gardneri*. *Tuber canaliculatum* differs in large (>50 μm long) ascospores (Gilkey 1954), while *T. linsdalei* has subglobose ascospores and a reticulum with numerous small meshes ("alveoli small and numerous"; Gilkey

1954). Long ellipsoid ascospores distinguish *Tuber longisporum* and *T. gardneri* from *T. microverrucosum*.

ITS sequence analyses (Fig. 1) support erection of *T. microverrucosum*. *Tuber microverrucosum* and *T. foetidum* grouped in the same clade with moderate support value (BS = 74, PP = 0.99), indicating that they are closely related but morphologically quite distinct.

Tuber huizeanum L. Fan & Yu Li, **sp. nov.** Fig. 2 e–i

MycoBank MB 563658

Differs from other *Tuber* species by its brown glabrous ascomata, brown colored gleba, and very broadly ellipsoid ascospores with a regular reticulum.

Type: China. Yunnan Province, Huize County, in soil under conifers, 28 Dec. 2011, Jin-Zhong Cao 513 (**Holotype**, BJTC FAN186).

Etymology: *huizeanum* (Lat.), referring to the type locality of the new species.

Ascomata 1.5–2.0 cm, subglobose, solid, surface smooth, glabrous, sometimes with a few furrows, white to yellow-white at first, light brown to brown at maturity. Odor slight, not pungent. Peridium 350–400 μm thick, two layers; outer layer 100–150 μm, pseudoparenchymatous, composed of subangular or subglobose cells mostly 10–25(55) μm, with slightly thickened and light yellowish walls, hypha-like hairs absent from the outermost cells; inner layer composed of intricately interwoven hyphae, hyaline, thin-walls, branch, septate, 2.5–5 μm, intermixed with a few of hyphae up to 10 μm in diam. Gleba brown at maturity, marbled with large and rare, branch white veins coming from the peridium. Asci subglobose, ellipsoid or irregular, hyaline, slightly thick-walled, 80–115 × 75–100 μm, sessile, 1–4-spored. Ascospores subglobose to very broad ellipsoid, a few of globose, light brown to yellow-brown at maturity, 27.5–42.5 × 25–40 μm, Q = 1.06–1.15, in 2–4-spored asci, and 50–55 × 42.5–45 μm, Q = 1.10–1.25, in 1-spored asci, excluding ornamentation; ornamentation regularly alveolate reticulum, 3–5 μm high, the meshes generally 4–8(10) across the spore width.

Additional material examined: **China**. **Yunnan Province**, Kunming, from the Kunming mushroom market. 20 Jan. 2011, Jin-Zhong Cao 107 (BJTC FAN144).

Comments — Three species from China are similar to the new species. *Tuber zhongdianense* has similarly colored ascomata and shaped ascospores as *T. huizeanum*. However, *T. zhongdianense* ascomata are obviously puberulent with two types of hairs and the ascospore reticulum is only 2.5 μm high (He et al. 2004). These two characters clearly distinguish the two species. *Tuber liui* differs by its large (38–71 × 27–40 μm) and ellipsoid to long ellipsoid ascospores (Xu 1999) and a peridium with hypha-like hairs. *Tuber latisporum* is differentiated by its white ascomata, blackish gleba, red brown ascospores, and a peridium covered with hypha-like hairs.

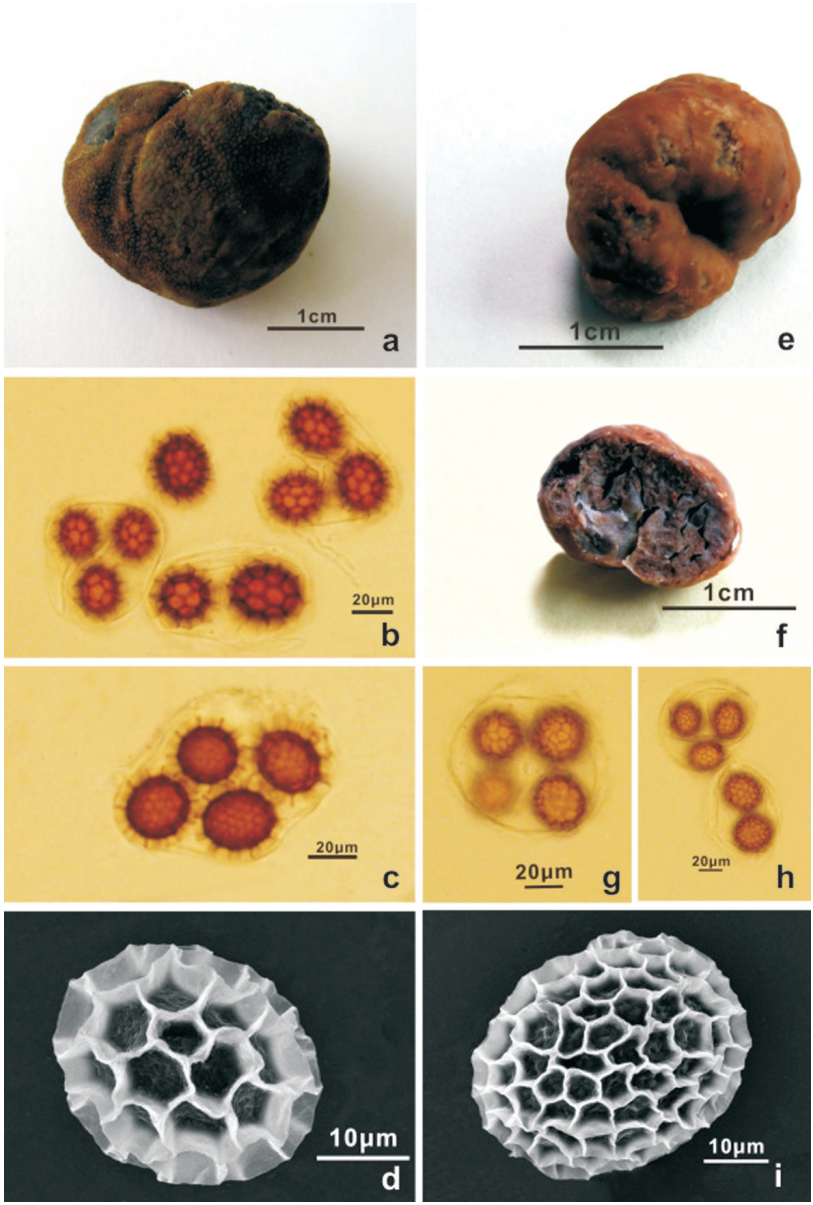

Fig. 2. ***Tuber microverrucosum*** (BJTC FAN142, holotype): a. Ascoma; b–c. Asci and ascospores observed under light microscope; d. Ascospore observed under SEM. ***Tuber huizeanum*** (BJTC FAN186, holotype): e–f. Ascoma; g–h. Asci and ascospores observed under light microscope; i. Ascospore observed under SEM.

The similar European *T. puberulum* can be separated by its ascomata with abundant surface hairs and globose ascospores. *Tuber borchii* has similar ascospores but differs by its dark chocolate brown mature gleba and puberulent peridium. The North American *T. separans* is distinguished by its inconspicuous glebal veins (Gilkey 1954).

Phylogenetic analyses (Fig. 1) support the erection of *T. huizeanum*. The morphologically similar *T. huizeanum* and *T. zhongdianense* form a sister relationship in the phylogeny but with low support (Fig. 1).

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